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The Chemical Behaviour of Heavy Metals Plays a Prominent Role in the Induction of Oxidative Stress

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It is often described that different environmental stress factors stimulate the production of reactive oxygen species and increase the activity of several enzymes quenching these radicals. The ascorbate–glutathione pathway is also involved in plant defence against oxidative stress. Therefore the effects of 2 metals (Cu, Zn) with different chemical behaviour were investigated on the enzymes of this pathway in the primary leaves of bean seedlings grown on hydroponics and supplied with a 50 μM concentration of both metals.

The results obtained demonstrate that the capacities of the enzymes involved in the ascorbate–glutathione pathway increase after metal application, indicating that they induce oxidative stress indeed. However striking differences in the relative induction time of these enzymes suggest that the chemical behaviour of the metals applied, plays an important role in the induction of oxidative stress as well as in the defence mechanism against it.

Keywords: Copper, zinc, *Phaseolus vulgaris*, primary leaves, antioxidative defence mechanism, ascorbate–glutathione pathway

INTRODUCTION

Copper and zinc are plant micronutrients that become phytotoxic at supra-optimal concentrations. Stunted growth, leaf epinasty and chlorosis are visible symptoms of strong metal toxicity. At lower metal concentration, these macroscopic symptoms are less pronounced or even absent, but cellular processes can be affected. At this level various avoidance mechanisms are distinguished: metal exclusion, translocation, complexation in the cytoplasm.[1] If avoidance is insufficient, free metal concentration increases and can cause oxidative stress. Since previous studies demonstrated the induction of several cellular antioxidative defence reactions in plants grown on metal contaminated substrates,[2,3] oxidative stress may be involved in metal phytotoxicity. The efficiency of the defence mechanisms depends on the nature, concentration and exposition time of the metal applied. In these

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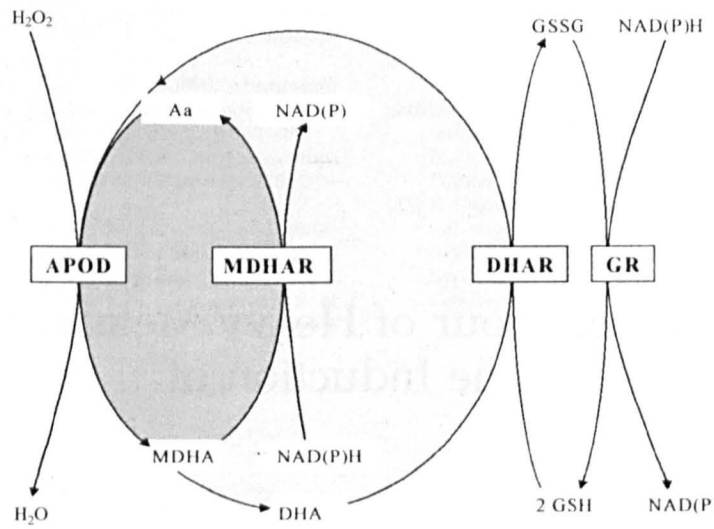

FIGURE 1 Ascorbate–glutathione pathway.

studies attention was generally focused on the induction of catalases, peroxidases, superoxide dismutases and $NAD(P)^+$-reducing enzymes.[1] However the ascorbate–glutathione pathway also participates in the defence against oxidative stress.

In the present study it was investigated whether copper and zinc affect the ascorbate–glutathione pathway in primary leaves (Figure 1). Since in contrast to zinc, copper easily performs one electron oxidoreduction reactions, the possible relation between the induction of this pathway and the chemical behaviour of the metal applied was also examined.

MATERIAL AND METHODS

Plant Material

Dwarf beans, *Phaseolus vulgaris* L. cv. Limburgse vroege, were grown under controlled environmental conditions (10 h light, 22°C, 65%RH, PAR = 165 $\mu mol\, m^{-2} s^{-1}$) on an aerated nutrient solution. Ten days after sowing, $CuSO_4$ or $ZnSO_4$ was added to the nutrient solution to a final concertration of 50 μM. Leaf samples were collected at different moments after metal application (0, 1, 5, 24, 48, 72, 96, 120, 168 h), frozen in liquid nitrogen and stored at −70°C.

Metal Analysis

The metal content of primary leaf tissue was determined by atomic absorption spectroscopy after microwave wet digestion of the dried material in supra-pure concentrated HNO_3.

Enzyme Assays

Enzyme capacities (activities in non-limiting conditions for substrate and coenzyme) were measured spectrophotometrically[4,5] on a crude leaf extract.

Statistical Analysis

The estimated values are the means of 9 samples taken from 3 independent experiments. The statistical analysis was based on the linear mixed model (MIXED procedure in SAS/STAT).[6]

RESULTS

Copper and Zinc Content in Primary Leaves after Root Metal Uptake

After application of 50 μM Cu to 10 days old seedlings, the Cu content in the primary leaves did not differ significantly compared to the controls until 24 h after the start of the treatment. Only at 48 h, the leaf Cu content of treated plants was twice the value of the controls (Figure 2a). The level still enhanced until the end of the treatment.

A significant rise of Zn in primary leaves was observed already 24 h after the start of the Zn treatment. A linear increase of the Zn content occurred till 72 h, where the Zn content reached a value about 4 times higher than the control (Figure 2b). This level remained constant till the end of the experimental period (168 h).

Increase of the Enzymes Constituting the Ascorbate–Glutathione Pathway

Table I shows the time after the start of the metal treatment, when significant enhancement of the enzyme capacities involved in this pathway was observed. After Cu treatment, glutathione reductase (GR) and monodehydroascorbate reductase (MDHAR) were already enhanced after 5 h. Increased dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APOD) capacities were observed after 24 and 48 h respectively. After Zn application no increase of GR capacity was observed, in contrast to Cu. DHAR capacity

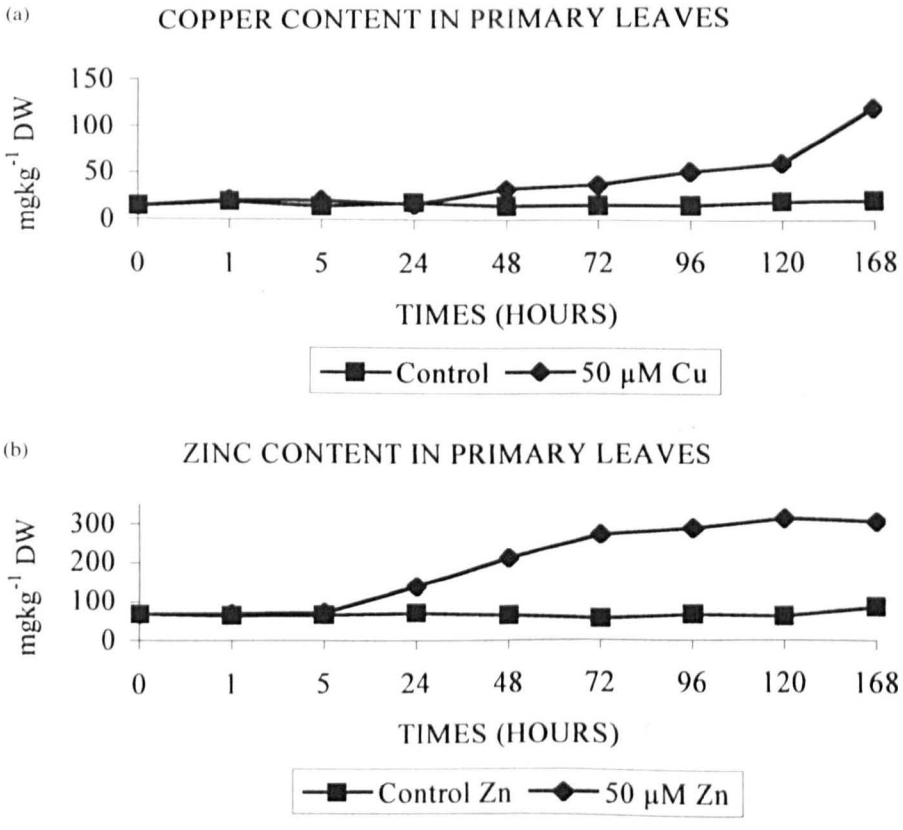

FIGURE 2 Evolution of copper (a) and zinc (b) content (mg kg^{-1} dry weight) in the primary leaves of *Phaseolus vulgaris* after metal application to 10 days old bean seedlings.

TABLE I Time (hours) after metal application necessary for significant increase of enzyme capacities

Enzyme	50 μM Cu (%)	50 μM Zn (%)
APOD	48 h** (186)	96 h** (172)
MDHAR	5 h** (130)	96 h** (275)
DHAR	24 h* (113)	24 h* (125)
GR	5 h** (135)	No increase

The numbers between brackets denote the % increase of the enzyme capacities of the treated plants with respect to the controls.
*$P < 0.05$;
**$P < 0.001$.

was increased first (24 h), followed by MDHAR and APOD capacities (96 h).

DISCUSSION

Metals are frequently observed to induce oxidative stress.[2,3] The impact of two metals with different chemical behaviour (Cu, Zn) was investigated on the enzymes constituting the ascorbate–glutathione pathway in the primary leaves of intact bean seedlings after metal uptake through the roots.

Roots are in direct contact with the metal applied to the nutrient solution, whereas these elements are mainly transported to the leaves in complexed form.[7] The capacities of GR, MDHAR and DHAR (Table I) already rose before any increase in leaf Cu content was observed (between 24 and 48 h). In contrast the capacities of three enzymes involved in the ascorbate–glutathione pathway were enhanced by Zn after uptake of this metal in the primary leaves. This suggests that within 5 h after Cu application to the roots, signal molecules are transported from the root to the primary leaves to activate the antioxidative defence mechanism. This activation may function to prevent the leaves from primary cellular damage caused by an excess of Cu later on in the experiment. This early response was not observed for Zn. This metal is mainly accumulated in the leaves as Zn-phosphate (Monceau, personal communication) which is highly insoluble. Therefore it can hardly interfere with the cell metabolism at low concentrations.

A further striking difference in the response to toxic amounts of Cu and Zn is the early increase of the GR capacity by Cu while Zn does not increase this enzyme at all. GR reduces glutathionedisulphide (GSSG) into glutathione (GSH). This metabolite might play a pivotal role in the defence mechanism against Cu toxicity. GSH is the major low molecular mass thiol compound in plants.[8] Thiols are important antioxidants and Cu has a high affinity for sulphydrylgroups.[9,10] The early increase of the GR capacity by Cu might be the result of: (1) GSH oxidation by reactive oxygen species produced by Cu itself and (2) GSH consumption for Cu detoxification by metal complexation. Zn neither interferes with one electron oxidoreductions as they occur in the Haber–Weiss reaction nor shows it high affinity for SH-groups.[11] Therefore the effect of Zn on GR capacity might be limited.

A third difference between the response to Zn and Cu is the relatively early increase of MDHAR capacity by the latter metal. Since Cu easily performs univalent oxidoreduction reactions, it oxidises ascorbate, yielding the monodehydroascorbate radical (MDHA). Buettner[12] showed that transition metals, like Cu, are able to mediate the formation of MDHA. Fast increase of MDHAR might prevent primary leaves from cellular oxidative damage caused by MDHA and Cu. Contrary to Cu, Zn is unable to perform monovalent oxidoreduction reactions which implies no direct formation of MDHA by Zn. Previous studies demonstrated a late increase of hydrogen peroxide content in the primary leaves of *Phaseolus vulgaris*[3] after Zn treatment. A non-enzymatic oxidation between ascorbate and hydrogen peroxide, yielding MDHA[12] might explain the late increase of the MDHAR capacity after a Zn treatment.

With Cu, DHAR capacity was enhanced later than MDHAR. This might be caused by the

spontaneous disproportionation of MDHA to yield ascorbate and dehydroascorbate.[13] After Zn treatment, on the contrary, DHAR was the first enzyme increased. Further investigation is required to clarify these results.

In both treatments APOD capacity was increased the last. A specific role for hydrogen peroxide in the signal transduction pathway may be therefore questioned. Although heavy metals are considered to induce oxidative stress, their mechanism of action is different and depends on their chemical behaviour. Analysis of the antioxidative metabolites (ascorbate, GSH) is in progress to further elucidate their mechanism of action.

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